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Women with inherited mutations in the BRCA2 gene have a very high lifetime risk of developing breast cancer. Previously, we developed a mouse with an exon 10/11 *Brca2* mutation by using standard gene targeting in embryonic stem cells. Unfortunately, these mice were embryonic lethals. Therefore, we have now generated mice carrying a *Cre-lox*P conditional *Brca2* mutated allele by flanking exon 27 with *lox*P sites. We predict that the site-specific recombination of *lox*P sites and deletion of exon 27 in this floxed *Brca2* allele by a *Cre* recombinase protein will disrupt basic functions of *Brca2* in DNA repair. The mammary-specific removal of *Brca2* exon 27 by Cre-mediated recombination *in vivo* is performed by crossing the homozygous floxed *Brca2* mice with MMTV-Cre transgenic mice. The formation of mammary gland tumors and altered mammary gland morphogenesis is anticipated. Viable homozygous $Brca2^{\Delta 27}$ animals, as well as various homozygous $Brca2^{\Delta 27}$ cell lines, have also been generated and should be extremely useful for testing proposed biological functions of *Brca2*. Mammary-specific *Brca2* (Δflox) mice should mimic women who have inherited a BRCA2 defect and later acquire a secondary somatic BRCA2 mutation in the breast and should therefore be a valuable animal model for mammary tumor development.

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Rickey A. Mallette 11/24/99

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Mammary-Specific Targeting of the *Brca2* Breast Cancer Susceptibility Gene in Mice

Introduction:

Approximately one in nine women will develop breast cancer in her lifetime. The breast cancer susceptibility gene BRCA2 is known to be responsible for a substantial portion of inherited breast cancer but very little is known about the basic mechanism of gene activation for BRCA2. The early embryonic lethality of *Brca2* null mice that we have previously generated impeded functional analyses of *Brca2* in normal mammary gland development and its role in neoplasia. We therefore proposed and have now generated mice carrying a conditional *Brca2* mutation whereby *Brca2* is disrupted specifically in the mammary tissue by gene targeting with the Cre-loxP system. We hope these mammary-specific *Brca2* (Δflox) mice will more closely mimic women that have inherited one defective breast cancer gene and develop a secondary mutation later in life in the breast. We hope to use these mice to define the phenotypes associated with the loss of *Brca2* function during normal and neoplastic development of the mammary gland. Studies with this conditional *Brca2* knockout mouse may help to clarify the effect of various environmental insults on breast cancer risk in a properly controlled environment without the genetic variation intrinsic to the human population. Viable homozygous $Brca2^{\Delta 27}$ animals, as well as various homozygous $Brca2^{\Delta 27}$ cell lines, have also now been generated and these resources will also be useful for *in vitro* biochemical studies designed to further identify the role of *Brca2* in DNA damage repair pathways and its role in mammary tumorigenesis.

Body:

Statement of Work summary: We have now completed the Tasks 1, 2, 3 of Technical Objective 1 in the Approved Statement of Work for this grant. The COOH-terminal *Brca2*-loxP targeting construct has been constructed and Southern blot analysis and PCR identified a properly targeted ES cell clone. Blastocyst injection and transfer has allowed the identification of chimeric mice capable of germline transmission of the flox allele and current crosses of these *Brca2* (flox) mice with MMTV-Cre transgenic mice is allowing the disruption of the *Brca2* locus to occur in the mammary gland specifically. From this training, I have been introduced to many of the emerging conditional knockout technologies including various cloning techniques, manipulation of ES cells, general animal husbandry and necropsy skills, and mammary gland whole mount analysis and histology methodology.

Specific Aim 1:

We have now generated a conditional knockout mouse model for *Brca2* that would allow the deletion of the final exon of the gene at a later time in the mammary

gland specifically. Through use of the *Cre-lox*P technology, a conditional *Brca2* knockout will allow us to generate mice that become defective for *Brca2* function specifically in the mammary gland during puberty. We predict that removal of the final exon and the polyA tail will disrupt *Brca2* function since a critically defined *Rad51* DNA binding domain has been identified in this exon (Sharan, *et al.* 1997; Mizuta, *et al.* 1997).

In the generation of this conditional *Brca2* mouse knockout, the mouse homologue of BRCA2 has been disrupted through the use of a targeting construct which has the final exon of the gene, exon 27, flanked by *lox*P sites. Double *lox*P oligonucleotides flanked by restriction site ends were generated to insert *lox*P sites into a Mfe 1 site in intron 26 and in a downstream region beyond the 3' untranslated region of *Brca2* and the Neomycin gene (Figure 1). A 5' targeting fragment consisting of a 4 kb EcoRI fragment containing exons 25, 26, and 27 was subcloned from a mouse BAC clone (McAllister, *et al.*, 1997). A 3 kb Nsi fragment containing the 3' untranslated region of homology beyond the *Brca2* stop codon and the putative position of the polyA tail was also subcloned and both fragments were inserted into a previously designed tk-pgkNeo targeting vector generously provided by Donna Bunch, NIEHS. Following linearization with a Sal I restriction site, this targeting vector was introduced into ES cells by electroporation. The electroporated cells were positively selected for the presence of Neomycin (Neo) and negatively selected for the absence of the thymidine kinase (TK) gene. A properly targeted ES cell clone (1F1) was then identified by both PCR analysis using *lox*P-specific primers and by Southern blot analysis.

To introduce this mutation into mice, C57BL/6J blastocysts were injected with the 1F1 cell line and transplanted into pseudopregnant CD-1 females. Fifteen generated chimeras were then used in matings to C57Bl/6J females and approximately half of the chimeras produced offspring with germline transmission of the *Brca2* floxed allele. Mice heterozygous for the floxed allele were intercrossed to determine the viability of the homozygous flox/flox genotype. The flox/flox genotype is represented at birth in the expected Mendelian ratios with respect to other genotypic classes and no obvious phenotype has been observed in these mice or in Flox/Δ11 mice (double heterozygous mice for the two *Brca2* mutations). The addition of the Cre recombinase protein will allow the site-specific recombination between loxP sites to occur removing exon 27 as well as the neomycin resistance gene.

Specific Aim 2:

The puberty-specific deletion of *Brca2* exon 27 in the mammary tissue has been performed by generating homozygous *Brca2* floxed mice ($B2F^{F/F}$) that have been crossed with an MMTV-Cre transgenic mouse strain ($MCD^{(Cre/+)}$) where Cre recombinase activity is restricted to mammary tissues by activation of a murine mammary tumor virus (MMTV) promoter with the onset of ovarian function during puberty (Wagner, *et al.* 1997). To more carefully define the predicted spatial and temporal expression pattern of the Cre transgene in these Cre transgenic mice, we have performed crosses between the $MCD^{(Cre/+)}$ strain and a Cre reporter mouse strain that carries a flox/STOP/flox/*LacZ*

cassette under the control of a ubiquitious ROSA26 promoter which is expressed in essentially all adult mouse tissues (Soriano, 1999). Cre-mediated lacZ expression can be detected by standard histological procedures in all cells of mice with the ROSA26 $MCD^{(Cre/+)}$ genotype where the Cre recombinase has been activated. These experiments are critical for defining the exact timing and tissue distribution of *Brca2* inactivation in this conditional knockout model. Analyses of ROSA26 LacZ Cre reporter mice confirm that the MMTV-Cre strain D transgene is expressed early during mammary ductal morphogenesis with good specificity (Figure 2).

Two crosses were required to generate female mice carrying the MMTV-Cre transgene and two floxed exon 27 *Brca2* alleles:

First Cross

$B2F^{F/F}$ x $MCD^{(Cre/+)}$ will generate 50% of offspring with the desired genotype:

$(B2F^{F/+})MCD^{(Cre/+)}$F1

Second Cross

$(B2F^{F/+})MCD^{(Cre/+)}$F1 x $B2F^{(F/F)}$ will generate 12.5% of offspring of desired female experimental genotype ($B2F^{(F/F)}MCD^{(Cre/+)}$ N2 females); 12.5% of offspring of desired female flox control genotype ($B2F^{(F/F)}MCD^{(+/+)}$ N2 females).

The initial experiments with these mice will involve comparing mammary tumor latency and incidence between mice that will have mammary-specific deletion of exon 27 *Brca2* with *Brca2* floxed control mice (with no Cre transgene present). Mice from each genotypic class will be observed for mammary tumor latency and incidence as well as for preneoplastic alterations at interim sac dates (2, 3, 6, 9, and 12 months of age). Early preliminary analysis of several $B2F^{(F/F)}MCD^{(Cre/+)}$ N2 females revealed no distinct morphological alterations in mammary gland morphology compared to $B2F^{(F/F)}MCD^{(+/+)}$ N2 female controls (Figure 3).

A conditional knockout for *Brca1* of a Cre-mediated excision of exon 11 in mouse mammary epithelial cells has now been generated utilizing these same $MCD^{(Cre/+)}$ mice and the resulting mice develop mammary tumors whose pathology is similar to human breast cancer (Xu, *et al.* 1999). These mutant mice also display abnormalities in mammary morphogenesis including increased apoptosis and abnormal ductal development. Based on these observations, we predict that our similarly constructed conditional *Brca2* mutant mice crossed with these same Cre transgenic mice will likewise be a valuable model for mammary tumorigenesis.

Because viable mutant *Brca2* mice were derived from mutations generated further downstream in exon 11 (Connor, 1997; Friedman, 1998), we predicted that animals homozygous for deletion of exon 27 of *Brca2* ($Brca2^{\Delta 27}$) would probably be viable at least to some degree and may very likely have an interesting phenotype. Transient

electroporation of 1F1 embryonic stem cells carrying a single floxed *Brca2* allele was therefore performed with a Cre-expression plasmid. The successful deletion of the floxed allele was generated in approximately 10% of these cells and the identification of $Brca2^{\Delta 27}$ ES cells was determined by using primers flanking the 5' and 3' loxP sites. These $Brca2^{\Delta 27}$ ES cells were injected into blastocysts as described previously. Eight out of nine generated pups derived from this single blastocyst injection were chimeric and germline transmission of the $Brca2^{\Delta 27}$ allele has now been obtained. Heterozygous crosses were recently performed to ascertain the phenotype of the homozygous $Brca2^{\Delta 27}$ animals. Intercrosses of $Brca2^{\Delta 27/+}$ animals indicate that mice with homozygous deletions are completely viable at birth. These *Brca2*-deficient mice are currently under observation for neoplastic development in all tissues. Alterations in normal growth and differentiation of mammary tissue in these *Brca2*-deficient female mice are being examined as well using whole mount analysis.

In parallel studies, mice homozygous for $Brca2^{\Delta 27}$ have also been generated by crossing $Brca2^{F}$ animals with MMTV-Cre Strain A mice. Unlike the MMTV-Cre Strain D, which we are using for the conditional knockout of *Brca2*, $Brca2^{F}$ alleles that pass through the female germline of MMTV-Cre Strain A are deleted completely in all tissues of the resulting offspring. Current breeding of these male $Brca2^{\Delta 27/\Delta 27}$ mice suggest that they may be partially or totally infertile. We have recently sacrificed a few of these male $Brca2^{\Delta 27/\Delta 27}$ to examine gametogenesis in these mice. We believe that these homozygous mutant mice with a complete deletion of *Brca2* throughout all tissues and cell types will be a valuable complement to the conditional knockout studies.

Specific Aims 3, 4, 5 (Future Studies):

Task 4 (Objective 1) and Task 1, 2, 3, 4, and 5 in Objective 2 of Approved Statement of Work: We will continue to monitor the effect of disruption of *Brca2* in the mammary gland for homozygous *Brca2* floxed mice ($B2F^{F/F}$) that have been crossed with the MMTV-Cre transgenic mouse strain ($MCD^{(Cre/+)}$) with or without the additional environmental insult of radiation. We are interested in studying the possible protective effect of pregnancy and/or lactation for mammary gland tumorigenesis as well. We have thus begun to initiate a series of experiments examining the effect of multiple pregnancies with or without lactation on the development of mammary gland tumors for these mice compared to virgin controls. The establishment of *Brca2* (flox) mouse lines on various inbred strain backgrounds has been initiated as well to examine genetic background effects to mammary carcinogenesis. We believe the susceptibility of the conditionals as well as the homozygous $Brca2^{\Delta 27}$ mice to mammary gland tumorigenesis may be enhanced by placing these animals on a *p53*-deficient background and we have therefore recently initiated these long–term studies. We also believe the homozygous $Brca2^{\Delta 27}$ may be particularly sensitive to radiation and plan to do a long-term study on the effects of radiation for these mice in the future. We have begun to isolate embryonic fibroblast cells from homozygous $Brca2^{\Delta 27}$ as well as generate homozygous *Brca2* (Δflox/Δflox) ES cells in order to begin to analyze the unique *in vitro* properties of these *Brca2*-deficient cells. We also hope to isolate and characterize mammary epithelial cells

from the homozygous *Brca2*$^{\Delta 27}$ and homozygous *Brca2* (Δflox/Δflox) conditional mice. We hope this distinct targeted population of cells might be useful as a resource to investigate various gene-environment interactions for *Brca2* deficieny using such currently developing technologies as cDNA microarray analysis.

Figure 1. *Brca2*(flox) Targeting Strategy

The *Brca2* (flox) targeted allele (B2F) is compared to the endogenous genomic *Brca2* gene (Wildtype *Brca2* Locus). The addition of the Cre recombinase protein will allow site-specific recombination to occur and removal of exon 27 as well as the neomycin resistance gene will result (B2D). Transient electroporation of ES cells with the Cre plasmid has now been successfully performed. To successfully remove Brca2 exon 27 *in vivo* in a tissue and temporal specific manner, the floxed *Brca2* mice were crossed with MMTV-Cre transgenic mice.

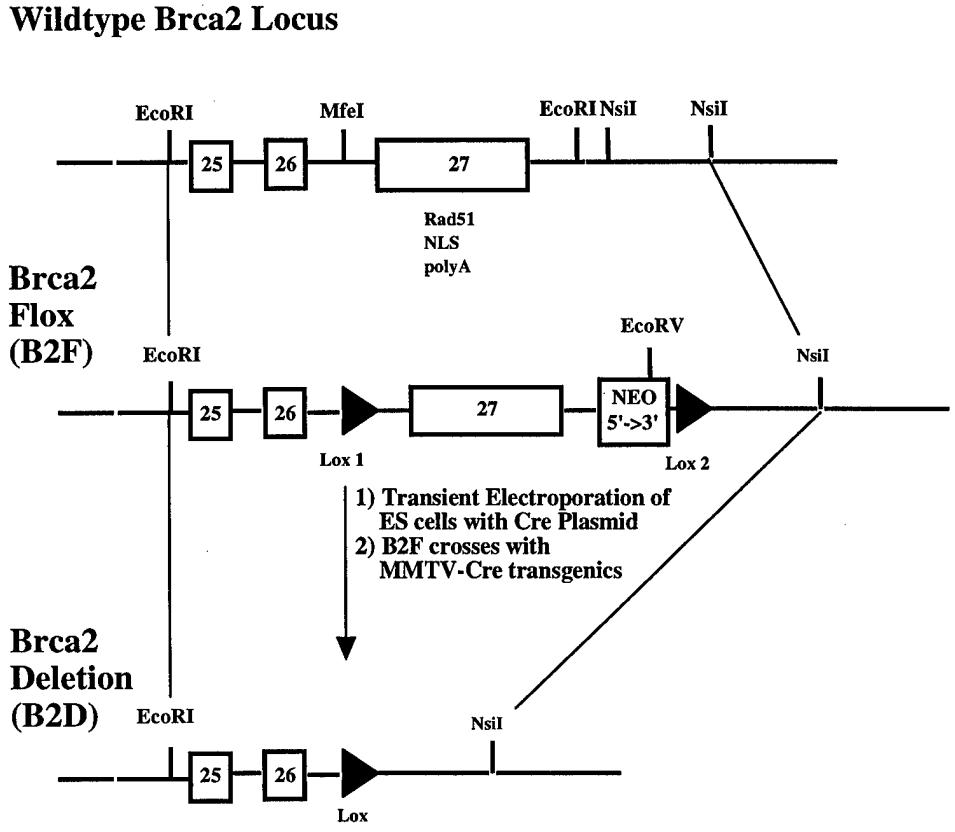


Figure 2. LacZ Staining in Mammary Gland Whole Mounts of MMTV-Cre x ROSA26LacZ-Cre reporter mice

Mammary glands from 8 week old offspring of MMTV-Cre mice crossed with ROSA26LacZ-Cre reporter mice were stained for LacZ to confirm the extent of Cre-mediated deletions in mammary epithelial cells carrying the Cre transgene. Beta-galactosidase activity was detected extensively in the mammary epithelial cells of mice that were LacZ+/Cre+ while no observable LacZ staining was detected in LacZ+/Cre- littermates.

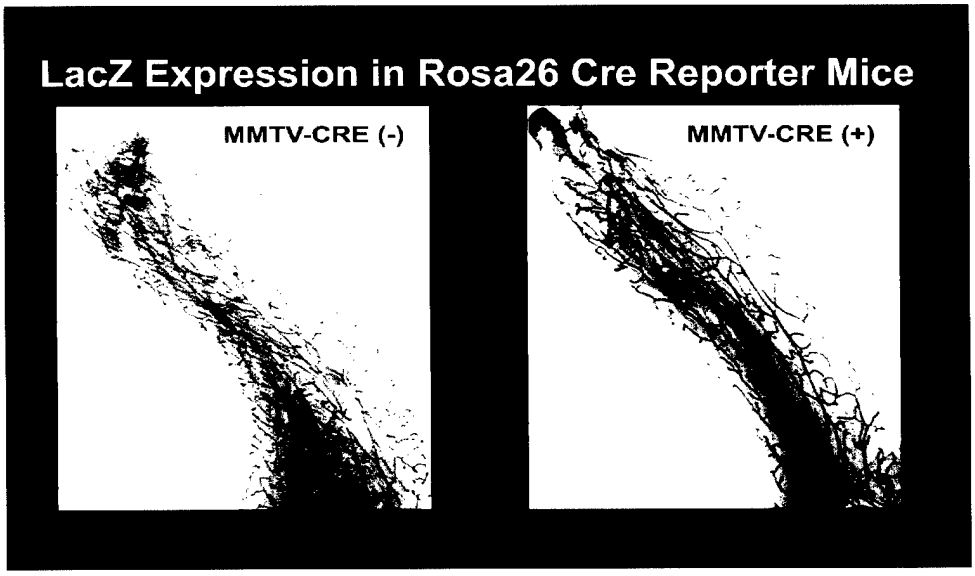
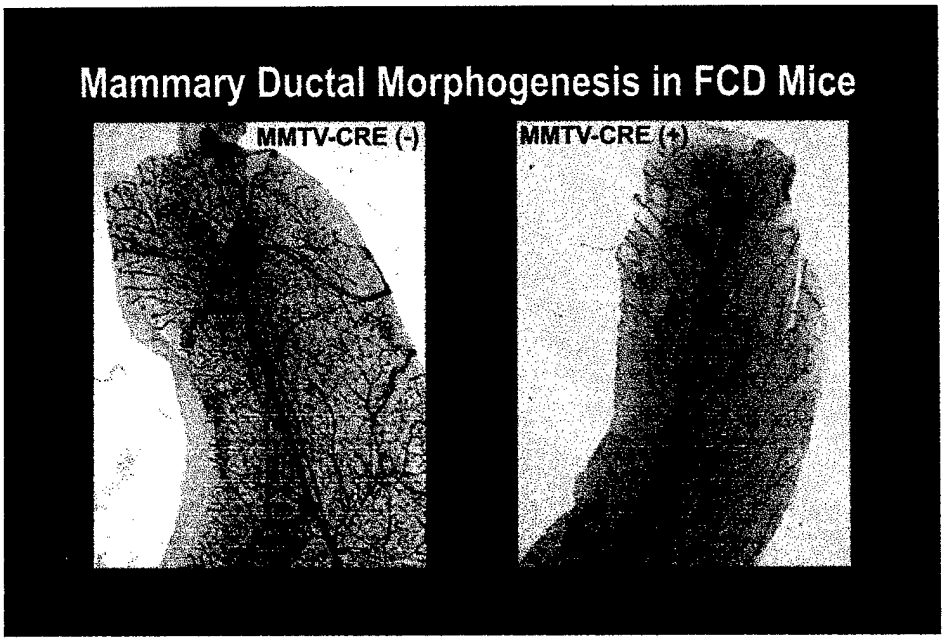


Figure 3. Mammary gland whole mounts of *Brca2* Flox x MMTV-Cre transgenics.

Analysis of ten week-old FCD animals (*Brca2* Flox/Flox x MMTV-Cre) revealed no obvious gross morphological abnormalities in the mammary gland for mice carrying the Cre transgene compared to those lacking Cre.



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Appendices:

Key Research Accomplishments:

**Brca2-loxP* targeting construct completed

*Properly targeted ES cell clone identified

*Generation of mice with germline transmission of the floxed *Brca2* allele

*Mammary-specific deletion of *Brca2* generated with *Brca2* floxed mice crossed with MMTV-Cre transgenic mice

*Specificity of MMTV-Cre strain D transgene confirmed with use of LacZ reporter mice

*Generation of homozygous $Brca2^{\Delta 27}$ mice

Reportable Outcomes:

Abstract entitled "Conditionally Targeted Deletion of the *Brca2* Breast Cancer Susceptibility Gene in Mice" presented at "Cancer Biology and the Mutant Mouse: New Methods, New Models, New Insights", AACR Special Conference, Keystone, Colorado. January 31-February 5, 1999.

Cited Abstract:

Cancer Biology and the Mutant Mouse

Conditionally Targeted Deletion of the *Brca2* Breast Cancer Susceptibility Gene in Mice
Kimberly A. McAllister, L. Michelle Bennett, Toni Ward, Jason Malphurs, N. Keith Collins, Sarah Hagevik, *Donna Bunch,*Gina Goulding, *Mitch Eddy, and Roger Wiseman. LMC, *LRDT, NIH, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709.

Inherited alterations in the human BRCA2 gene confer a profound predisposition to breast and ovarian cancer. Previously, we used gene targeting with embryonic stem cells to generate mice with a mutation that disrupts exons 10 and 11 of the Brca2 gene. Mice that are homozygous for this mutation exhibit embryonic lethality and this has impeded functional analyses of *Brca2* during normal and neoplastic development in the mammary gland and other adult tissues. Therefore, we have generated mice carrying a conditional *Brca2* mutation by flanking exon 27 with *lox*P sites. Prior studies have shown that this COOH terminal domain of *Brca2* interacts with Rad51 (Sharan, S. *et al.*, Nature 386:804-810, 1997) and cells that lack exon 27 of *Brca2* are hypersensitive to gamma-radiation (Morimatsu, M. *et al.*, Cancer Research 58: 3441-3447, 1998). Several approaches will be taken to remove *Brca2* exon 27 by *Cre*-mediated recombination *in vivo* including: 1) crosses of floxed *Brca2* mice with transgenic mice expressing the *Cre* recombinase under the control of a tissue specific promoter; 2) direct injection of adenovirus-*Cre* expression vectors into developing mammary glands of floxed *Brca2* mice; and 3) *in vitro* infection of floxed mammary epithelial cells with adenovirus-*Cre* expression vectors followed by transplantation of these cells into cleared mammary fat pads. Transient electroporation of embryonic stem cells carrying the floxed *Brca2* allele with a *Cre*-expression plasmid yielded multiple clones with site-specific deletion of exon 27. Paired cell lines that either express wild-type *Brca2* or Brca2$^{\Delta 27}$ (*e.g.* embryonic stem cells, fibroblasts, mammary epithelial cells) should be extremely useful for testing proposed biological functions of *Brca2* such as its role in maintenance of genome integrity through DNA repair and recombination pathways. Our ultimate goal is to use conditional *Brca2* gene disruption to develop mouse models for neoplastic development. These mammary-specific *Brca2*$^{\Delta 27}$ mice should mimic women who have inherited a BRCA2 defect and acquire a secondary somatic BRCA2 mutation in breast tissue later in life.